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Intracellular pH of Cotton Embryos and Seed Coats during Fruit Development Determined by ^{31}P Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT

The pH of the phosphate-containing compartments of developing cotton seed coat and embryo tissues was determined by means of ^{31}P nuclear magnetic resonance spectroscopy. The pH values of these tissues varied as a function of developmental age. From 27 to approximately 38 days postanthesis, a strong pH differential existed between the two tissues; the seed coat was up to 1.4 pH units more acid than developing cotton embryos. The pattern of pH values found with this technique agrees with pH values of tissue homogenates in distilled water. The results confirm an earlier suggestion that seed coat cells are more acidic than embryo cells during key developmental stages of the seed. The pH differential between these two tissues causes abscisic acid to diffuse from seed coats to embryos against its apparent concentration gradient to prevent viviparous germination, despite a higher abscisic acid concentration in the embryo.

Recent studies (8) indicate that immature cotton embryos accumulate ABA from seed coats by diffusion, apparently against its concentration gradient. This movement seems to be driven by a large pH differential between the two tissues during development. Evidence for this difference in intracellular pH is largely indirect. Aqueous extracts of seed coats were up to 0.75 pH units more acid than those of embryos, but the interpretation of this difference was unclear. Extraction of whole tissues necessarily causes mixing of subcellular compartments, and this mixing might result in an alteration in the measured pH. Here we report studies of intracellular pH of intact seed coats and embryos, using ^{31}P NMR spectroscopy. The results of this study confirm the existence of a large pH differential between developing cotton seed coat and embryo during that developmental stage when cotton embryos will germinate if cultured in media lacking ABA.

MATERIALS AND METHODS

Cotton (a glandless strain of *Gossypium hirsutum* L. cv Coker 100A) plants were grown in the field in Phoenix, AZ, or in a growth chamber illuminated with metalarc bulbs. Flowers were tagged on the day of anthesis and fruits (bolls) were harvested at various maturities. They were rapidly transported to the NMR Laboratory on ice. For NMR experiments, 12 to 18 developing ovules were removed, dissected into embryo and seed coat, and transferred to a 10 mm NMR tube containing 2 ml 2 mM Mes (pH 5.0) and 1.0 ml D_2O. The embryos were placed in the NMR tube intact, but seed coats were chopped first with a razor blade into pieces approximately 1 to 2 mm in diameter. A second set of bolls was harvested and the seed coats and embryos homogenized in 1 ml distilled water per seed coat or embryo. The pH was determined in the resulting suspension with a combination glass pH electrode (8).

The ^{31}P NMR analysis was performed at 161.98 MHz with a Bruker[1] AM 400 FT-NMR spectrometer, equipped with an Aspect 3000 data system. Transients of 4,096 data points were acquired using a sweep width of 16,666 Hz, a 16 μs pulse width (90°=22 μs), and a recycle delay of 100 ms. An exponential multiplication factor of 5 to 25 Hz, and zero-filling to 8192 points was used prior to Fourier transformation. The number of transients per spectrum varied from 220 to 1850 and spectra were acquired without proton decoupling. The deuterium NMR signal from the solvent was used both for field/frequency locking and for adjusting homogeneity. A coaxial capillary tube containing 0.5 M methylenediphosphonic acid, adjusted to pH 8.9 with Tris buffer, was placed in the NMR tube in some assays and chemical shifts were expressed in relation to this compound. Locking could usually be accomplished in less than 20 s. Chemical shifts were easily reproducible to better than ±0.05 ppm for identically treated samples.

Standard curves relating chemical shift to pH were prepared by grinding seed coat and embryo tissue in an ice-cold mortar, squeezing the homogenate through cheesecloth and centrifuging the resulting liquid to remove particles. The pH of this solution was then adjusted to various values with 0.5 M NaOH or HCl (14) and ^{31}P NMR spectra obtained as for the intact tissue samples. Separate curves were prepared for embryo and seed coat tissues. These spectra did not change over extended periods (4 min–2h) after grinding.

In order to reduce possible effects of anoxia (11, 12, 15), NMR sampling time was kept to a minimum. Typical acquisition times for embryos were from 0.6 to 2 min and 2.7 to 5.7 min for seed coats.

RESULTS AND DISCUSSION

Phosphorus NMR spectra were taken from immature cotton seeds beginning 28 d after anthesis and continuing until maturity (desiccation and fruit dehiscence). This period of cotton seed development encompasses the time during which embryos will germinate if removed from the fruit (5, 8). Spectra for developing

[1] Mention of a trade name does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval over other products that may also be suitable.

embryos from any age within this interval show a pronounced single peak of ^{31}P resonance (Fig. 1). No resonance peaks corresponding to cytoplasmic Pi signals (12, 13, 15) were observed in any of the approximately 25 embryo spectra analyzed; very similar spectra were observed when a single embryo was analyzed (data not shown). In contrast, the spectra of similarly aged seed coats often showed indications of cytoplasmic P compartments at approximately −15 ppm (Fig. 1), where the resonance of cytoplasmic glucose 6-P and cytoplasmic Pi would be expected. However, noise levels in our short scans made precise interpretations difficult. Ultrastructural examination of embryos of these ages revealed large masses of lipid and protein bodies, without providing clues to the relative volumes of cytoplasm and vacuole (3, 4). We are unaware of published microscopic studies of developing cotton seed coat tissue that might help explain the difference in these spectra.

Tissue pH (*i.e.* the pH of the dominant Pi-containing compartment) was determined by comparing the chemical shift of the ^{31}P peak to that of tissue extracts titrated to known pH values. For any given pH, the chemical shift of embryo extracts was slightly greater than that of seed coat extracts (Fig. 2). The difference between these standard curves could result from differences in other electrolytes in the two tissues (14). Using the standard curves developed for each tissue, we found that the pH of seed coat cells was considerably lower than that of embryo cells (Fig. 3, upper panel). The difference was about 1.4 pH units at 28 d after anthesis, decreasing to zero at 42 d after anthesis (just prior to dehiscence). This very large, age-dependent differential of pH was reflected in the pH values of aqueous extracts of the tissues (Fig. 3, lower panel). The extracts also displayed a large differential at younger ages, decreasing to zero around 42 d after anthesis. However, the maximum difference in this case was about 0.8 pH units (Fig. 3, lower panel), only approximately one-half of the maximal pH differential observed by NMR (Fig. 3, upper panel). Hendrix and Radin (8) earlier reported a maximum differential of 0.75 pH unit in aqueous extracts of the two tissues, and a very similar trend with development. The difference in these two measurements may be due to the compartmental mixing which occurs in tissue homogenization.

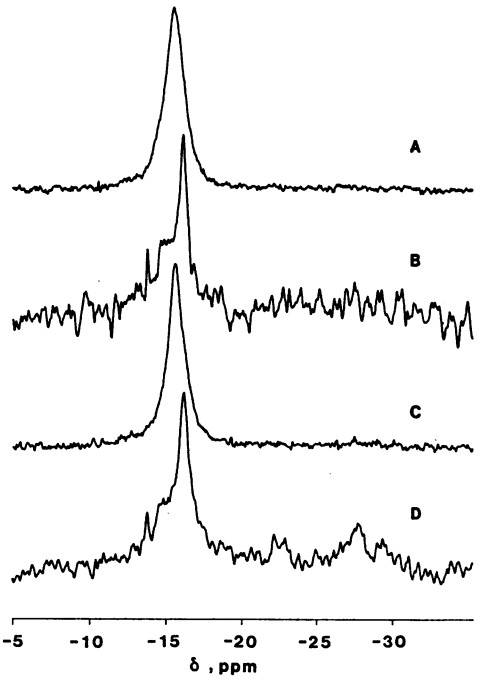

FIG. 1. Typical 161.98 MHz ^{31}P-NMR spectra obtained from developing cotton seed coat (B, D) and embryo (A, C) tissues, 35 d (A, B) and 29 d (C, D) from anthesis. Spectra were obtained over a period of 5.4 min for seed coats and 0.6 min for embryos, in 2 mM Mes (pH 5.0). The chemical shifts observed for the major peaks are: A, −15.578 ppm; B, −16.151 ppm; C, −15.625 ppm; and D, −16.202 ppm.

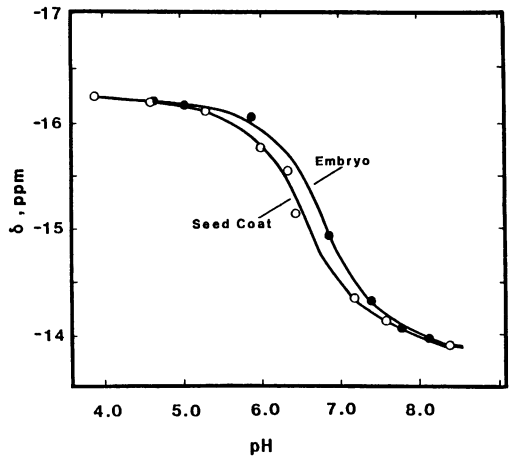

FIG. 2. Calibration curves for chemical shift (δ) *versus* pH for cotton embryo and seed coat tissues. Clarified homogenates of tissues were adjusted to various pH values with small amounts of HCl or NaOH and their ^{31}P-NMR spectra determined as for the intact tissue samples.

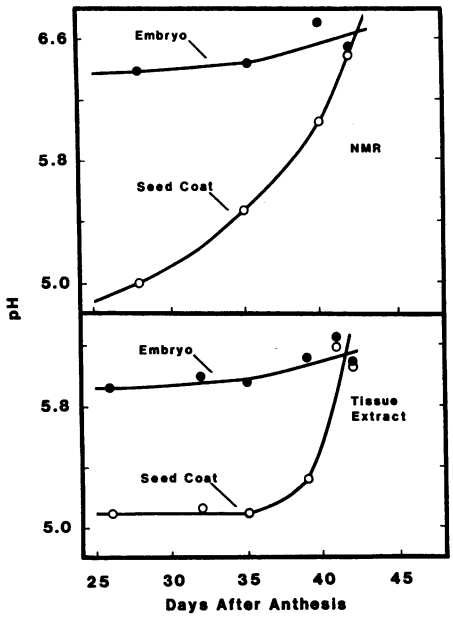

FIG. 3. Effect of development upon the pH of developing cotton seed coat and embryos by ^{31}P-NMR (upper panel) and by combination glass pH electrode (lower panel) in aqueous extracts.

The resonance peaks of these embryo spectra (Fig. 1) are slightly broader than those from seed coats or other tissues (15). The relative width of the embryo signal could be due to phytate in these cells. Phytate, a major P-component in mature seeds, has a distinctive and broad NMR signal in the same region of the spectrum as the embryo ^{31}P signal (Fig. 4). Homogenizing embryos, however, caused the ^{31}P signal to become as sharp as the signal from homogenized seed coats (Fig. 4). Because seed coats do not accumulate phytate, this result argues against phytate as the signal broadening agent in developing cotton embryos. Also, adding 15 mg phytate to homogenized embryos did not significantly broaden their ^{31}P spectra (not shown). Neither homogenization nor addition of phytate changed the area of the ^{31}P peak for cotton embryos (data not shown). The peak narrow-

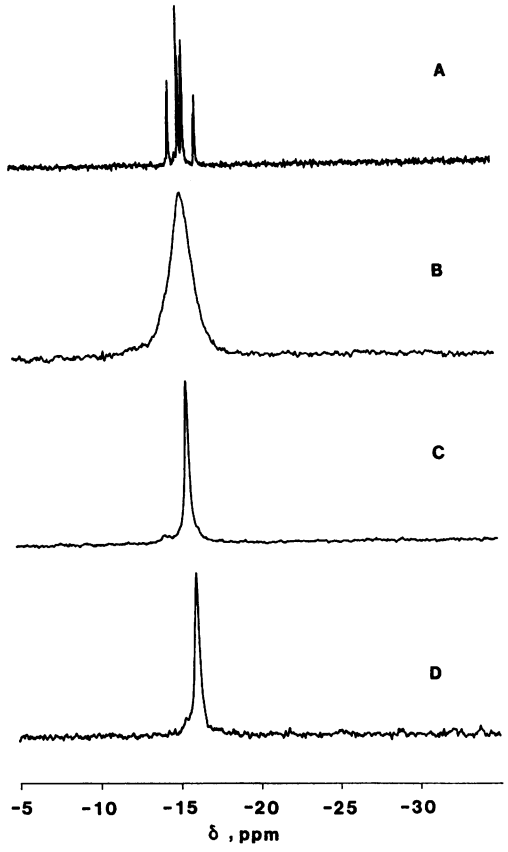

FIG. 4. Spectra obtained from 50 mg phytate (A), 18 intact embryos (B), 17 homogenized embryos (C), and 18 homogenized seed coats (D). The phytate spectrum was determined on calcium phytate dissolved in HCl and titrated to pH 5 with NaOH. All spectra were acquired without proton decoupling.

ing upon embryo homogenization also minimizes presumed interference from other solutes such as paramagnetic ions as they would be present in the homogenized extract as well. The most likely cause of peak broadening in the embryo spectra is cellular or structural heterogeneity.

In earlier work (8) most of the ABA in these tissues was found in a compartment with a relatively long half-time of exchange. It was thus termed 'vacuolar,' in analogy with the slowly exchanging compartment in inorganic ion efflux experiments. The identity of this compartment was, and is still, uncertain. The vacuole is a possible identity for this compartment, since it would have the size, pH, and presumably sufficient Pi to give rise to the observed signals. The literature does not suggest the vacuole as a repository for ABA, instead assigning it to chloroplasts (9). However, both tissues in this study lack chloroplasts. The pH of the embryo 'vacuoles' (Fig. 3) indicates that they might serve satisfactorily as alkaline traps for ABA, much in the manner of chloroplasts in other tissues.

Anoxia is known to decrease cytoplasmic pH of root tips (1, 12, 13). We did not perfuse these samples with oxygenated buffer during spectrum acquisition. However, anoxia should have not been a problem for two reasons. First, the chemical shift of vacuolar Pi is relatively resistant to anoxia (1, 15). Second, the time for the data acquisition was kept very short. The strong signal from embryos allowed useful spectra to be obtained in considerably under 1 min. The Pi resonance peak from cotton seed coats was much weaker, but useful spectra could be obtained from this tissue within about 5 min. Scanning a sample of seed coats for 1.1, 2.7, and 5.5 min produced no noticeable drift in the Pi peak (*cf.* 1) from cotton seed coats (data not shown). Five min represented a good compromise between the need for long runs for noise suppression, and the need for short incubations to avoid anoxia. These times are much shorter than frequently used in other laboratories (10, 15, 16).

In earlier work (8), we found that cotton embryos accumulate ABA during the period of their development in which they will germinate *in vitro* if cultured without ABA. The ABA concentration in the embryo during this period exceeds that in the seed coats and there is no symplastic connection between these two structures. Transport of this phytohormone to the embryo must therefore occur by diffusion, apparently against its concentration gradient. Kaiser and Hartung (9) had earlier postulated that the protonated ($ABA \cdot H$) but not the anionic (ABA^-) abscisic acid molecule could move readily across membranes. This mechanism leads to accumulation of ABA in the more alkaline compartments of a tissue (6, 7). The hypothesis of Kaiser and Hartung could explain our results if a pH differential of the proper sign and magnitude were to exist between the two tissues (*i.e.* if the embryos were significantly more alkaline than the seed coats). Although aqueous extracts of embryos were considerably more alkaline than those of the seed coats (8), the relationship between pH *in vivo* and the pH of the extracts remained unclear. Our ^{31}P-NMR spectra now demonstrate the existence of the large pH differential between intact embryos and seed coats in developing cotton seeds, as earlier inferred from the pH of aqueous extracts. Thus, these data provide strong support for the control of ABA movement by pH gradients in germinable immature seeds. A similar explanation has been recently invoked to explain the level of ABA in developing *Phaseolus* embryos (2). Mechanisms for maintenance of this large difference in $[H^+]$, and its role in other physiological processes (*e.g.* assimilate transport from seed coat to embryo) remain undetermined.

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